Lipid Peroxidation and Cellular Damage in Extrahepatic Tissues of Bromobenzene-Intoxicated Mice

ALESSANDRO F. CASINI, MD, MARCO FERRALI, PhD, ALFONSO POMPELLA, MD, EMILIA MAELLARO, PhD, and MARIO COMPORTI, MD

From the Istituto di Patologia Generale dell'Università di Siena, Siena, Italy

The mechanisms of bromobenzene toxicity in extrahepatic tissues of mice were studied. Kidney, lung, heart and brain were examined. As observed in this as well as in a previous report for the liver, bromobenzene intoxication caused a progressive decrease in the glutathione content of all the tissues examined. Cellular damage (as assessed by both biochemical determinations and histologic observations) appeared after 6 hours in the case of the kidney and the heart and after 15 hours in the case of the lung. Lipid peroxidation (as assessed by the tissue content of malonic dialdehyde, a parameter correlating with both the diene conjugation absorption and the amount of carbonyl functions in cellular phospholipids) was found to occur at the same times at which cellular damage was observed or even before. As in the case of bromobenzene-induced liver injury, when the individual values for cell damage obtained at 15–20 hours were plotted against the corresponding glutathione contents, a severe cellular damage was generally observed when the glutathione levels reached a threshold value (3.0–0.5 nmol/mg protein). Such a glutathione threshold was also observed for the onset of lipid peroxidation. Glutathione depletion and lipid peroxidation are therefore general phenomena occurring not only in the liver but in all the tissues as a consequence of bromobenzene poisoning. The possibility that lipid peroxidation is the cause of bromobenzene-induced damage to liver and extrahepatic tissues is discussed. (Am J Pathol 1986, 123:520–531)

IT HAS BEEN known for a long time that the administration of bromobenzene and other halogenated aromatic hydrocarbons to rats and mice results in the production of hepatic necrosis.[1–4] The latter is believed to be mediated by the covalent binding of bromobenzene metabolites to target macromolecules of the liver cell.[5–8] However, since these toxins markedly deplete the liver cell of glutathione (GSH), another reasonable mechanism of cellular damage can be represented by the development of peroxidation of membrane lipids in a cell depleted of one of the major defenses against the oxidative stress. Lipid peroxidation has been in fact observed in isolated hepatocytes treated with a number of GSH-depleting agents,[9,10] as well as in primary cultures of hepatocytes intoxicated with bromobenzene.[11] Also, lipid peroxidation, as assessed by ethane evolution, was increased in mice acutely intoxicated with acetaminophen.[12,13]

Recent studies from our laboratory[14] have shown that following bromobenzene or iodobenzene administration to mice liver necrosis develops only when the hepatic GSH depletion reaches a threshold value. Lipid peroxidation, measured by different methods, was found to occur at a very extensive level, and the process developed when the hepatic GSH depletion reached the same critical values as those observed for liver cell death. The treatment of the intoxicated animals with the antioxidant Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a lower homolog of vitamin E, completely prevented both lipid peroxidation and necrosis, while not changing at all the extent of the covalent binding of bromobenzene metabolites to liver protein.[14] This result supports, therefore, the view that lipid peroxidation represents the major mechanism responsible for the bromobenzene-induced liver necrosis.

It has been demonstrated that bromobenzene and other related aromatic hydrocarbons, in addition to

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Address reprint requests to Prof. Mario Comporti, Istituto di Patologia Generale dell'Università di Siena, Via del Laterino 8, 53100 Siena, Italy.

causing hepatic necrosis, can also exert toxic effects on extrahepatic tissues. These include the lung, where a necrosis of the bronchiolar epithelium has been reported,[15] and the kidney, in which these toxins produce necrosis of the proximal convoluted tubules.[16] Furthermore, it has been shown that following bromobenzene administration, bromobenzene metabolites are covalently bound to cellular proteins of lung and kidney and to a lesser extent heart and other extrahepatic tissues.[15-18] Therefore, it was investigated whether bromobenzene induces in extrahepatic tissues alterations similar to those seen in the liver, that is, whether the decrease in cellular GSH and the onset of lipid peroxidation are general phenomena occurring, besides in the liver, in many other tissues after the administration of bromobenzene to the living animal.

Materials and Methods

Male NMRI albino mice (Ivanovas GMBH, Germany) weighing 20–30 g and maintained on a pellet diet (Altromin-Rieper, Bolzano, Italy) were used. As reported in a previous study,[14] mice resulted more susceptible to bromobenzene intoxication than rats, in which the hepatic GSH depletion after bromobenzene treatment was less pronounced. This different susceptibility has also been reported by others for acetaminophen intoxication.[19] The animals were fed a liquid glucose diet (20% glucose) for 2 days before the intoxication according to the protocol followed by Wendel and Feuerstein.[13] This was done to decrease the hepatic GSH content. This regimen, in fact, decreased hepatic GSH by about 50% as compared with that in laboratory chow fed animals and increased the frequency of liver necrosis.

Bromobenzene (C. Erba, Milan, Italy) mixed with two volumes of mineral oil was administered intragastrically under light ether anesthesia at the dose of 15 mmol/kg body wt. Control mice received mineral oil alone. All the animals were starved after the intoxication.

In this study lipid peroxidation was assessed by measuring the tissue content of malonic dialdehyde (MDA), one of the end products of lipid peroxidation. Tissue samples were homogenized in ice-cold trichloracetic acid (TCA) (1 g tissue plus 1 ml 10%, wt/vol, TCA plus 8 ml 5%, wt/vol, TCA, or equivalent amounts) in an Ultra Turrax tissue homogenizer. After centrifugation, a volume of the supernatant was added to an equal volume of 0.67% (wt/vol) thiobarbituric acid, and the mixture was heated at 100 C for 10 minutes. The absorption spectrum was then recorded over 480–600 nm. The spectrum was quite similar to that obtained with an MDA standard produced by the acid hydrolysis of 1,1,3,3-tetraethoxypropane and run under the same conditions. The MDA concentration was calculated from the absorption at 532 nm (absorption maximum) of the difference spectrum (intoxicated minus control animals) with the use of a molar extinction coefficient of 1.56×10^5, as reported by others[20] and also recalculated from our standards.

In preliminary experiments run with the livers of bromobenzene-intoxicated mice, lipid peroxidation was measured by various methods, and it was ascertained that the method measuring the tissue content of MDA correlated with the other methods (Casini et al, unpublished results): in particular, with the detection of the diene conjugation absorption in cellular phospholipids, as done by Casini et al,[21] and with the evaluation of the amount of carbonyl functions originating from the peroxidative breakdown of unsaturated fatty acids in cellular phospholipids, as done by Benedetti et al.[22]

Liver damage was assessed by measuring the glutamate-pyruvate transaminase (SGPT) activity (optimized UV enzymatic method, C. Erba, Milan, Italy). Lung damage was assessed by measuring the lactate dehydrogenase (LDH) activity (optimized UV enzymatic method, C. Erba, Milan, Italy) in the lung lavage fluid.[23] The lung lavage was performed by cannulating the trachea immediately after the sacrifice of the animal. Kidney damage was evaluated by measuring the blood urea nitrogen (Urea test, Sclavo, Siena, Italy). Heart damage was assessed by measuring the serum creatine-kinase MB (CK-MB) activity UV test, Merck, Darmstadt, Germany). Because, as will be reported in the Results section, lipid peroxidation occurs, besides in heart, in skeletal muscle after bromobenzene poisoning, we measured, besides the serum CK activity, the serum CK-MB activity because the latter isoenzyme is reported to be specific of the heart. In the method for the assay of this isoenzyme the CK-M subunits are inhibited by the specific antibody. The antibody present in the kit is prepared against the human CK-M subunits. The antibody, however, is very probably working against the mouse CK-M subunits as well, because the serum CK-MB activities assayed with the kit in mice are by far lower than those of total serum CK.

Tissue damage was also assessed by the histologic examination of the various tissues. Tissue samples were fixed in Bouin and embedded in paraffin wax. Hystologic sections (4 μ thick) were prepared by standard techniques and stained with hematoxylin and eosin.

Results

First of all, we have confirmed our previous findings[14] that bromobenzene intoxication results in the onset of lipid peroxidation in the liver and elevation of SGPT

Table 1—Time Course of Hepatic Glutathione (GSH) Depletion, Liver Damage (SGPT), and Lipid Peroxidation (Hepatic Content of Malonic Dialdehyde, MDA) After Bromobenzene Poisoning

	Time after intoxication					
	0 min	3 hours	6 hours	9 hours	15 hours	18–20 hours
GSH (nmol/mg protein)	21.6 ± 2.2 (11)	5.0 ± 0.9*[b] (4)	4.2 ± 0.5 (5)	3.5 ± 0.7 (4)	1.9 ± 0.7 (15)	2.3 ± 0.3 (23)
SGPT (U/l)	32 ± 7 (10)	33 ± 11 (4)	35 ± 9 (5)	50 ± 21 (4)	112 ± 42 (15)	1457 ± 514*[a] (23)
MDA (pmol/mg protein)	–	0 (4)	0 (5)	3.3 ± 3.1 (4)	18.3 ± 15.0 (15)	523.3 ± 142.5 (21)

Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Significantly different from the 0-minute value: [a]$P < 0.1$, [b]$P < 0.002$.

activity when the hepatic GSH depletion reaches a threshold value. Table 1 shows the time course of hepatic GSH depletion, liver damage (as assessed by SGPT), and lipid peroxidation (as measured by the MDA content of the liver) after bromobenzene poisoning. Elevation of both SGPT and MDA was observed after 9 hours, even though at 9 hours the GSH content was already decreased by 84%, as compared with that of glucose-fed nonintoxicated animals. As noticed in the previous study,[14] a large individual variation was observed in the sensitivity of the animals to bromobenzene, as shown by the large dispersion of the SGPT and MDA levels (Table 1). There was, however, a readily evident relationship between the different parameters of

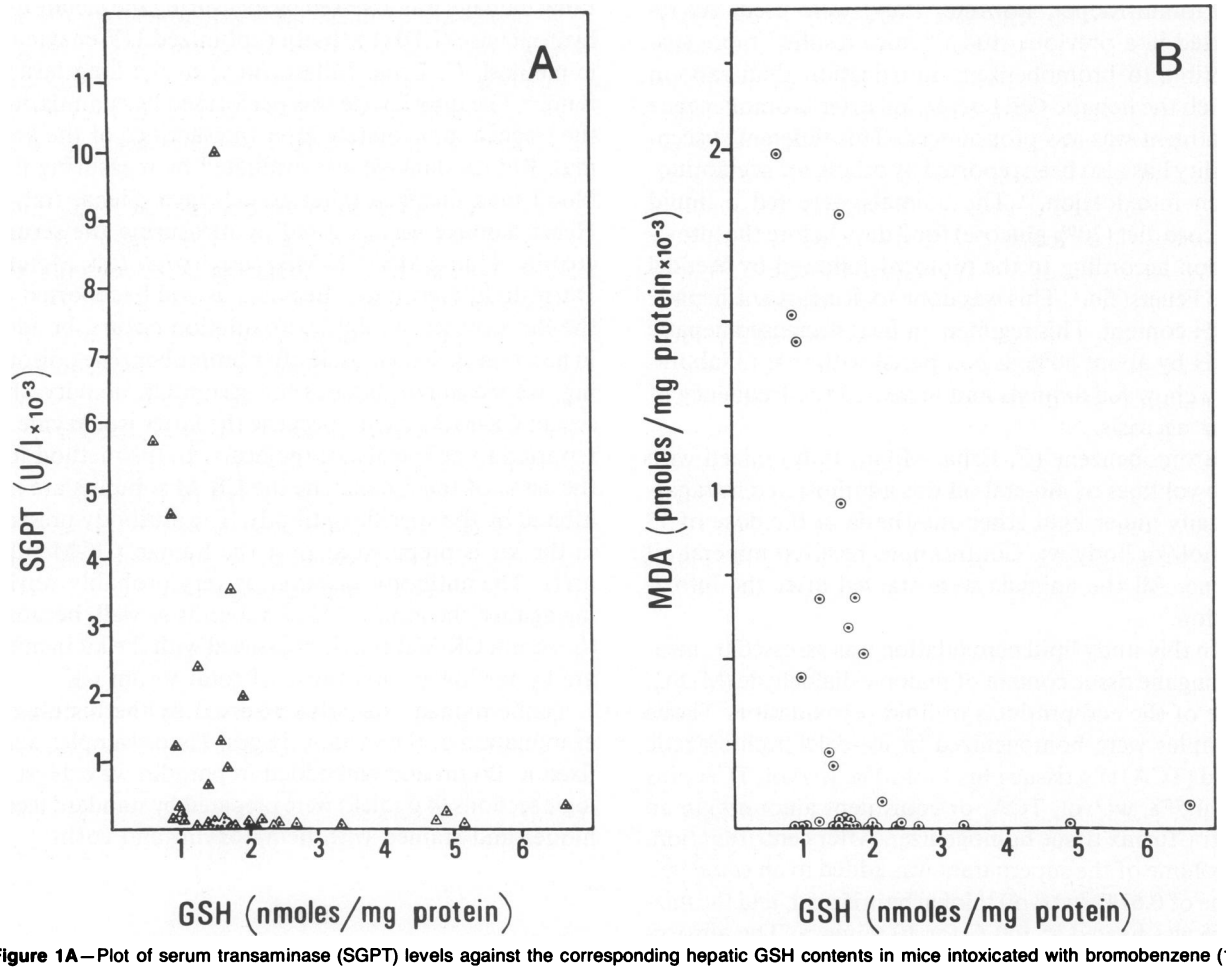

Figure 1A—Plot of serum transaminase (SGPT) levels against the corresponding hepatic GSH contents in mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth). **B**—Plot of lipid peroxidation values (malonic dialdehyde contents of the liver) against the corresponding hepatic GSH contents in mice intoxicated with bromobenzene. In 2 cases (4.76 and 5.17 nmol GSH/mg protein) lipid peroxidation was not measured. The values reported in **A** and **B** were obtained at 15–20 hours after bromobenzene poisoning.

the response to bromobenzene when the values for the individual animals were graphed together. The plot of the individual values obtained at 15–20 hours for transaminase (Figure 1A) or MDA levels (Figure 1B) against the corresponding hepatic GSH contents indicated that lipid peroxidation and elevation of SGPT occurred only when the hepatic GSH levels reached a threshold limit (2.2–0.9 nmol/mg protein). By plotting the log of the parameters studied, negative correlations were found between hepatic GSH concentrations, on the one hand, and SGPT levels ($P < 0.05$) or MDA values ($P < 0.05$), on the other hand.

Therefore, we investigated whether, in the same animals, bromobenzene induces in extrahepatic tissues alterations similar to those seen in the liver.

Bromobenzene-Induced Kidney Damage

Table 2 shows a time course study of the alterations (GSH decrease, cellular damage, and lipid peroxidation) produced by bromobenzene in the kidney. The GSH content of the kidney, which at 0 time was comparable to that of the liver, decreased progressively with the intoxication time up to 15 hours. The blood urea nitrogen level (kidney damage) increased after 9 hours. Similarly, lipid peroxidation (MDA content of the kidney), which was absent through 6 hours, was found to occur from the ninth hour onward. The mean MDA value was, however, much lower than that obtained for the liver.

As in the case of bromobenzene-induced liver damage, when the individual blood urea nitrogen values obtained at 15–20 hours were plotted against the corresponding GSH contents (Figure 2A), high levels of blood urea nitrogen were evident only when the GSH level reached a threshold value (2.2–0.8 nmol/mg protein). Such a GSH threshold was also evident for the onset of lipid peroxidation (Figure 2B). The GSH contents of the kidney were inversely correlated with both blood urea nitrogen levels ($P < 0.001$) and MDA values ($P < 0.01$), as assessed by plotting the log of the parameters studied. Thus, the results obtained for the kidney are similar to those obtained for the liver.

The histologic observations confirmed the above data. A severe tubular necrosis was visible in mice with high levels of blood urea nitrogen (over 10 mg/100 ml). The histopathologic effects of bromobenzene were particularly marked in the corticomedullary region, in which the tubular cells appeared in various stages of degeneration (Figure 3). Many proximal and distal convoluted tubules were markedly dilated and filled with acidophilic casts, sometimes containing cellular debris (Figure 3). A complete denudation of the tubular epithelium was observed in some areas. No appreciable changes were observed in the glomeruli.

Bromobenzene-Induced Lung Damage

Table 3 shows a time course study of the alterations (GSH decrease, cellular damage, and lipid peroxidation) produced by bromobenzene in the lung. The GSH content of the lung, which at 0 time was lower than that of liver and kidney, decreased markedly during the first 6 hours after the intoxication; subsequently, the decrease was more gradual. Lipid peroxidation appeared after the third hour. The LDH activity in the lung lavage fluid (cellular damage) was increased at 18–20 hours.

When the individual values obtained at 15–20 hours for both LDH (Figure 4A) and MDA (Figure 4B) were plotted against the corresponding GSH contents, the values appeared more dispersed than in the case of the kidney, so that critical threshold limits of GSH could not be defined. No significant correlations were found by plotting the log of LDH or MDA values against the log of GSH concentrations. It was, however, generally evident that high levels of lipid peroxidation and cellular damage are accompanied by low levels of GSH.

Examination of the lung by light microscopy revealed morphologic differences in the small airways between control and treated animals (Figure 5). The epithelial

Table 2—Time Course of Glutathione (GSH) Depletion, Cellular Damage (Blood Urea Nitrogen Level) and Lipid Peroxidation (Malonic Dialdehyde Content, MDA) in Kidney After Bromobenzene Poisoning

	Time after intoxication					
	0 min	3 hours	6 hours	9 hours	15 hours	18–20 hours
GSH (nmol/mg protein)	23.9 ± 2.0 (8)	16.1 ± 1.4*[a] (4)	13.2 ± 1.0*[b] (5)	7.7 ± 1.1*[c] (4)	1.3 ± 0.2 (15)	1.9 ± 0.3 (33)
Blood urea nitrogen (mg/100 ml)	31.1 ± 3.5 (8)	30.2 ± 3.1 (4)	29.4 ± 2.5 (5)	36.0 ± 6.3 (4)	–	80.5 ± 7.3*[c] (25)
MDA (pmol/mg protein)	–	0 (4)	0 (5)	33.5 ± 33.5 (4)†	15.7 ± 7.6 (15)	76.9 ± 14.7 (33)

Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Significantly different from the 0-minute value: [a]$P < 0.05$, [b]$P < 0.005$, [c]$P < 0.002$.

† Of 4 animals, only 1 showed elevated MDA levels (134.1 pmol/mg protein); no detectable MDA was found in the others.

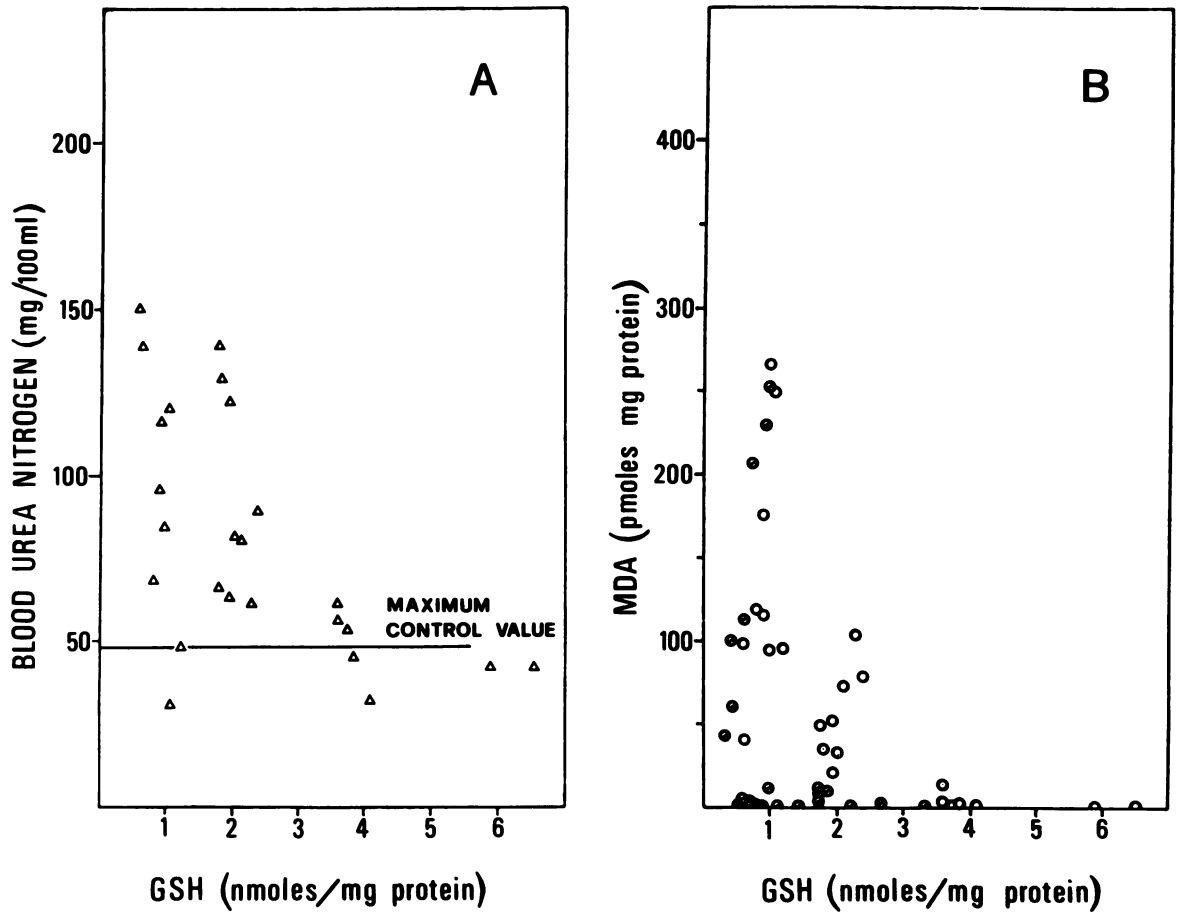

Figure 2A—Plot of blood urea nitrogen levels against the corresponding GSH contents of the kidney in mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth). **B**—Plot of lipid peroxidation values (tissue contents of malonic dialdehyde) against the corresponding GSH contents in the kidney of mice intoxicated with bromobenzene. In some cases (∅) blood urea nitrogen was not measured. The values reported in **A** and **B** were obtained at 15–20 hours after bromobenzene poisoning.

surface of terminal bronchioles showed focal areas of cellular necrosis and degenerative changes characterized by cytoplasmic vacuolation, nuclear condensation, and flattening of columnar nonciliated (Clara) cells. In some bronchioles necrotic columnar cells appeared sloughed into the lumen, and the basement membrane was either denuded or covered by flattened, abnormal-looking ciliated cells. No appreciable changes were observed in bronchi and lung parenchima.

Bromobenzene-Induced Heart Damage

Table 4 shows a time course study of the alterations (GSH decrease, serum CK-MB levels, and lipid peroxidation) produced by bromobenzene in the heart. The GSH level, which at 0 time was much lower than that of the other organs examined, decreased linearly with the intoxication time. Cellular damage (as assessed by the serum CK-MB activity) and lipid peroxidation (MDA content of the heart) appeared after 6 hours.

When the individual CK-MB values at 15–20 hours were plotted against the corresponding GSH contents (Figure 6A), higher levels of serum CK-MB activity were generally evident in correspondence to lower GSH levels. The same was observed for the onset of lipid peroxidation (Figure 6B). In this case, too, no significant correlations were found between GSH contents of the hearts and CK-MB or MDA values.

Figure 7 shows that bromobenzene poisoning results in the onset of lipid peroxidation in the skeletal muscle,* too, even if the MDA values are generally lower than those obtained with the heart. A GSH threshold was evident for the onset of lipid peroxidation. The GSH contents of the skeletal muscle were inversely correlated with MDA values ($P < 0.001$), as assessed by plotting the log of the parameters. The serum CK ac-

* The sartorius and gracilis muscles of the hind paw were examined. They were taken 20 hours after bromobenzene poisoning.

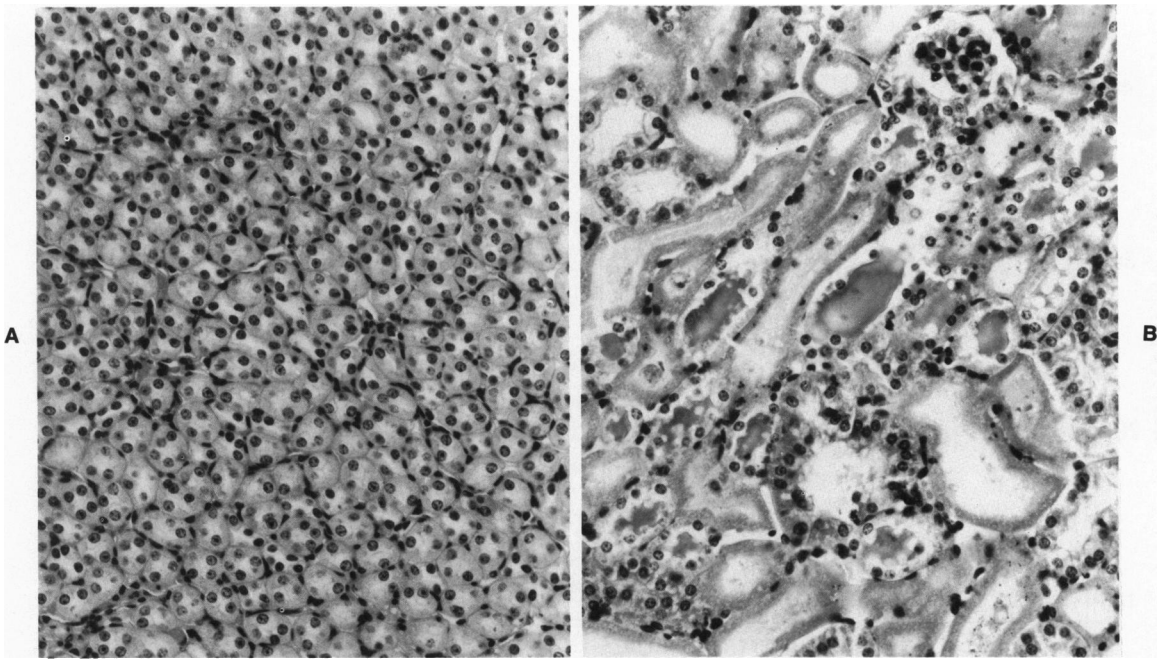

Figure 3—Paraffin sections of kidneys stained with hematoxylin and eosin. (×80) **A**—Corticomedullary area from a control mouse, showing a normal appearance. **B**—Corticomedullary area from a mouse intoxicated with bromobenzene (15 mmol/kg body wt, by mouth) and sacrificed 20 hours later. The section shows dilatation of tubular lumens, some of which appear filled with acidophilic casts and cellular debris. Areas of degeneration and necrosis are visible in the tubular epithelium.

tivity, which can derive from the damage of both the heart and the skeletal muscles, was increased by three to four times in the bromobenzene poisoned mice at 20 hours after the intoxication (data not shown).

The histologic examination of the heart (Figure 8) occasionally revealed areas of recent infarction in the intoxicated mice (18–20 hours after the intoxication).

Bromobenzene-Induced Brain Damage

The brain of the mice was divided into two sections, ie, forebrain and hindbrain (the latter including cerebellum), which were analyzed separately. Table 5 shows that bromobenzene intoxication produces GSH depletion and lipid peroxidation in both forebrain and hindbrain. The analysis was carried out at 18–20 hours after the administration of the toxin. As can be seen, the GSH depletion was higher in hindbrain (−55%) than in forebrain (−39%) of the intoxicated animals ($P < 0.002$).

When the individual values for lipid peroxidation in both forebrain and hindbrain were plotted against the corresponding GSH values (Figure 9), it appeared that lipid peroxidation developed in forebrain only when the GSH depletion reached a threshold value (2.0–1.5 μmol/g wet tissue). A good negative correlation was found between the log of the two parameters studied ($P < 0.001$). A GSH threshold could not be clearly defined in hindbrain, which also showed more resistance to the development of lipid peroxidation than the fore-

Table 3—Time Course of Hepatic Glutathione (GSH) Depletion, Cellular Damage (LDH Activity in Lung Lavage Fluid), and Lipid Peroxidation (Malonic Dialdehyde Content, MDA) in Lung After Bromobenzene Poisoning

	Time after intoxication					
	0 min	3 hours	6 hours	9 hours	15 hours	18–20 hours
GSH (nmol/mg protein)	15.3 ± 0.6 (10)	8.3 ± 0.5*[b] (4)	5.7 ± 0.3 (5)	4.0 ± 0.4 (4)	2.9 ± 0.2 (15)	2.7 ± 0.3 (15)
LDH in lung lavage fluid (U/l)	83 ± 2 (6)	89 ± 15 (4)	93 ± 8 (5)	90 ± 12 (4)	108 ± 16 (15)	194 ± 55*[a] (5)
MDA (pmol/mg protein)	–	14.4 ± 14.4 (4)†	48.7 ± 21.8 (5)	27.8 ± 11.7 (4)	23.5 ± 6.0 (15)	88.3 ± 25.1 (15)

Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Significantly different from the 0-minute value: $^{a}P < 0.05$, $^{b}P < 0.002$.

† Of 4 animals, only 1 showed elevated MDA levels (57.8 pmol/mg protein); no detectable MDA was found in the others.

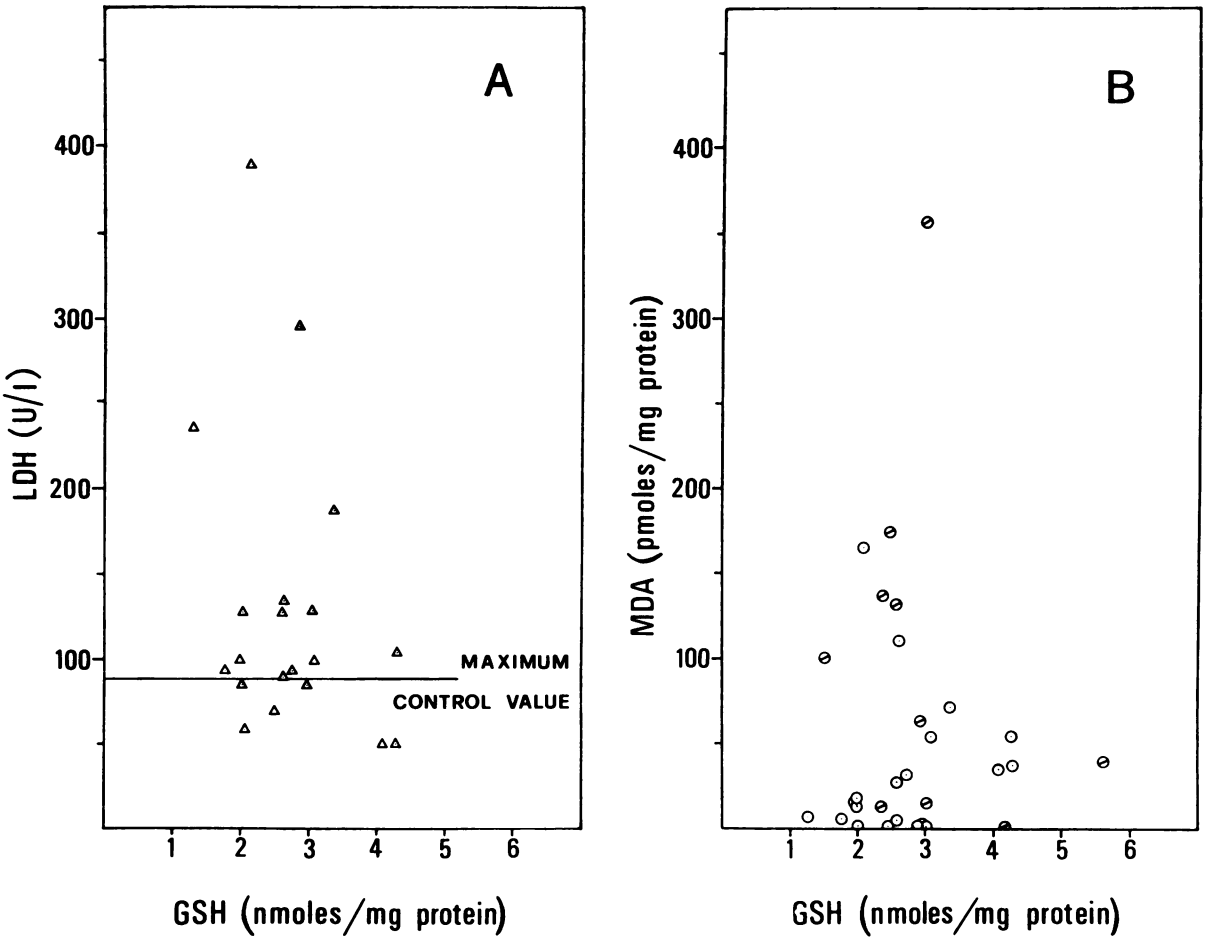

Figure 4A—Plot of LDH activities in the lung lavage fluid against the corresponding GSH contents of the lung in mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth). **B**—Plot of lipid peroxidation values (tissue contents of malonic dialdehyde) against the corresponding GSH contents in the lung of mice intoxicated with bromobenzene. In some cases (⊘) LDH activity was not measured. The values reported in **A** and **B** were obtained at 15–20 hours after bromobenzene poisoning.

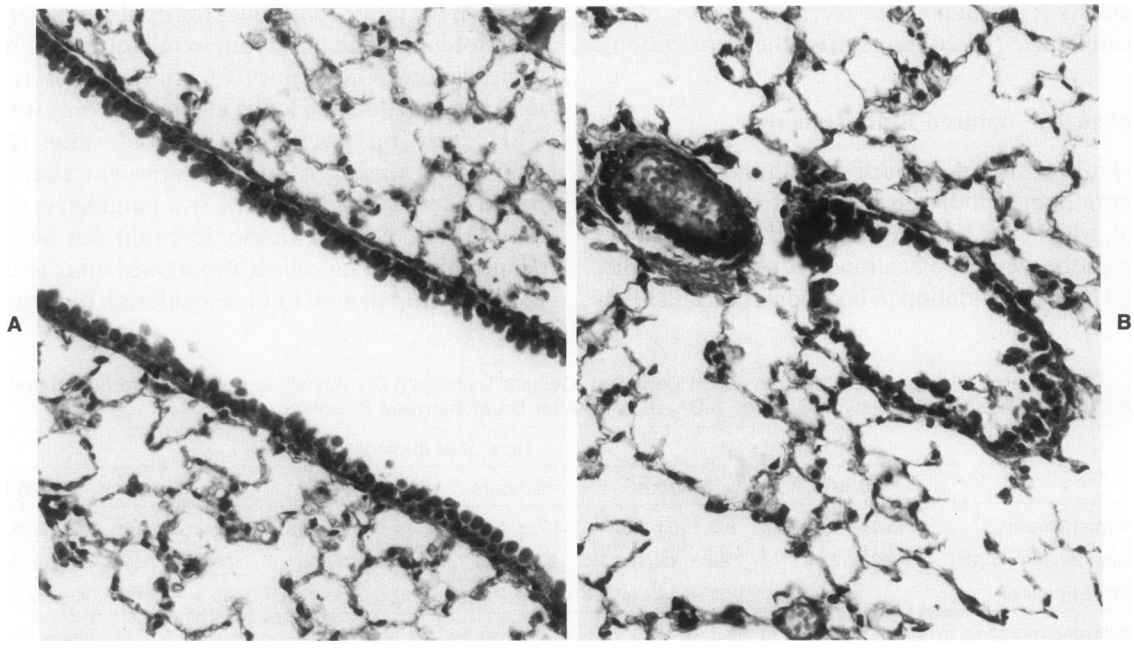

Figure 5—Paraffin sections of lungs stained with hematoxylin and eosin. (×80) **A**—Bronchiolar epithelium from a control mouse showing a normal appearance. **B**—Bronchiolar epithelium from a mouse intoxicated with bromobenzene (15 mmol/kg body wt, by mouth) and sacrificed 20 hours later. Note the presence of focal cellular necrosis. Bronchiolar lumen was delimited by flattened and vacuolated cells. Cellular debris are present in luminal spaces.

Table 4—Time Course of Glutathione (GSH) Depletion, Cellular Damage (Serum CK-MB Activity) and Lipid Peroxidation (Malonic Dialdehyde Content, MDA) in Heart After Bromobenzene Poisoning

	Time after intoxication					
	0 min	3 hours	6 hours	9 hours	15 hours	18–20 hours
GSH (nmol/mg protein)	7.3 ± 0.4 (11)	5.6 ± 0.3*[b] (3)	4.8 ± 0.3*[d] (5)	2.8 ± 0.4 (4)	1.4 ± 0.1 (15)	1.2 ± 0.2 (18)
CK-MB (U/l)	278 ± 70 (8)	219 ± 79 (4)	280 ± 49 (5)	801.2 ± 466.7 (4)†	1095 ± 252*[c] (6)	817 ± 214*[a] (18)
MDA (pmol/mg protein)	–	0 (4)	0 (5)	0.75 ± 0.75 (4)†	1.9 ± 0.2 (6)	99.9 ± 23.4 (18)

Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Significantly different from the 0-minute value: $^{a}P < 0.1$, $^{b}P < 0.05$, $^{c}P < 0.005$, $^{d}P < 0.002$.

† Of 4 animals, only 1 showed detectable MDA levels (3 pmol/mg protein) and elevated serum CK-MB activity (2187 U/l).

brain. This may be due to the different composition in the cells and fibers of the two sections of the brain, which in turn may result in a different capacity to either metabolize bromobenzene or conjugate its metabolites with GSH.

In the animals in which marked GSH depletion and lipid peroxidation occurred in the brain, cerebellar symptoms (action tremors) were noted.

Discussion

The present study demonstrates that bromobenzene intoxication induces GSH depletion and lipid peroxidation in the liver as well as in many extrahepatic tissues: kidney, lung, heart, skeletal muscle, brain. In all these tissues the MDA content was found to be increased several hours after the intoxication, although to a lesser

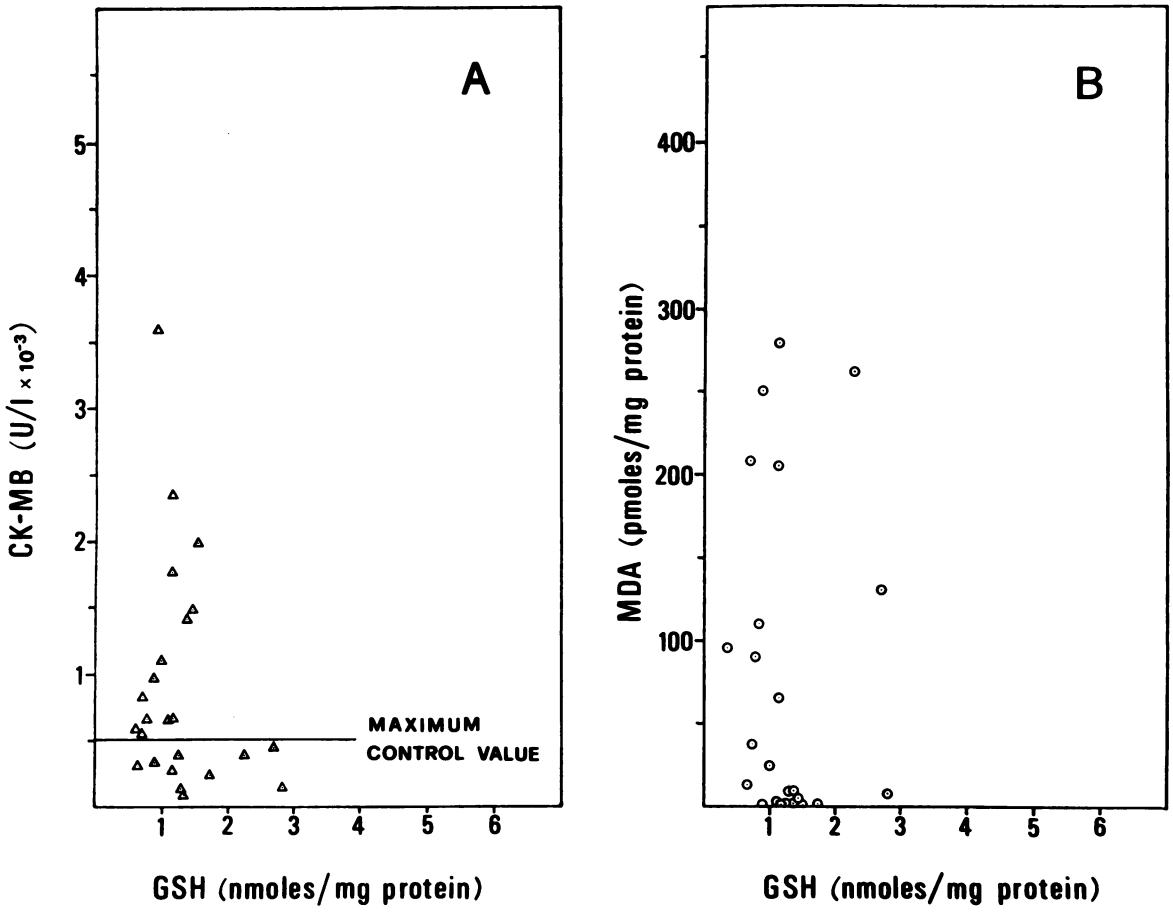

Figure 6A—Plot of serum creatine-kinase MB (CK-MB) activities against the corresponding GSH contents of the heart in mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth). **B**—Plot of lipid peroxidation values (tissue contents of malonic dialdehyde) against the corresponding GSH contents in the heart of mice intoxicated with bromobenzene. The values reported in **A** and **B** were obtained at 15–20 hours after bromobenzene poisoning.

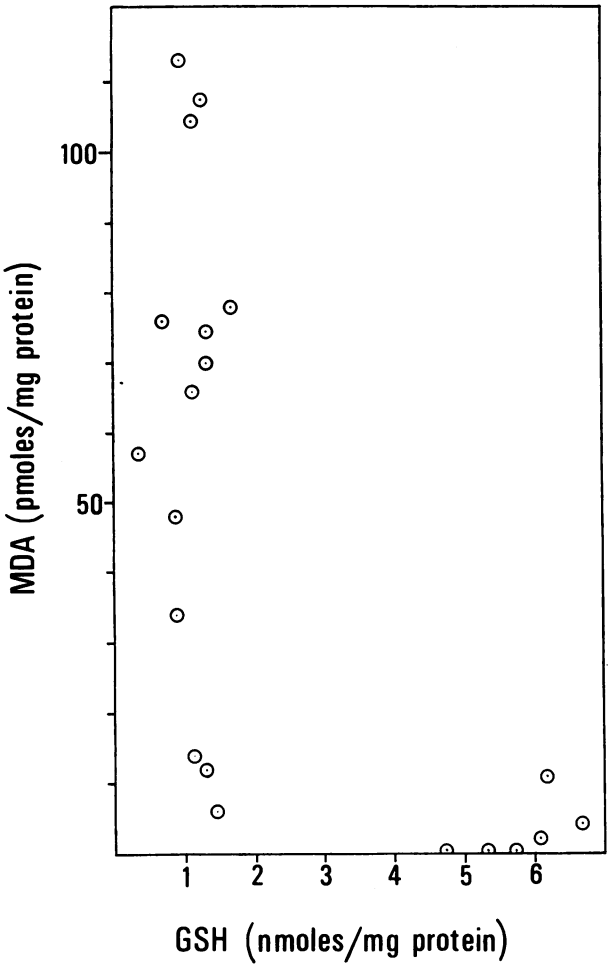

Figure 7—Plot of lipid peroxidation values (tissue contents of malonic dialdehyde) against the corresponding GSH contents in the skeletal muscle of mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth) and sacrificed 20 hours later.

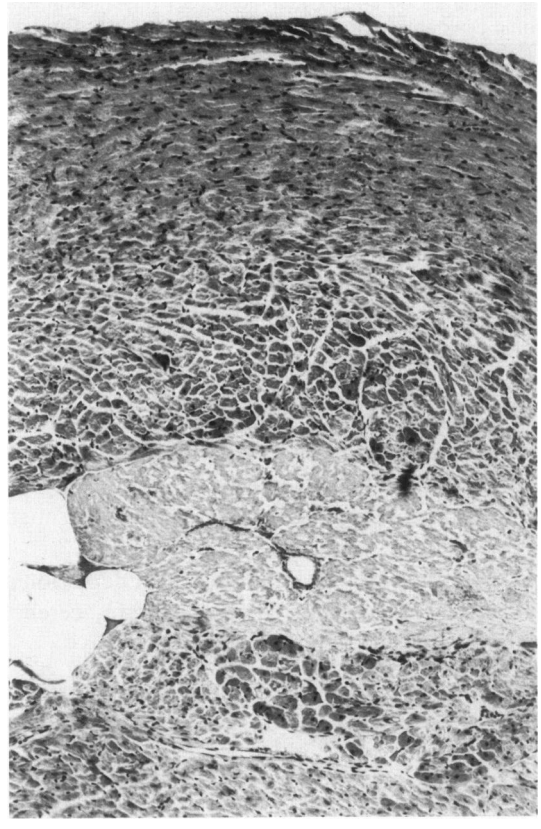

Figure 8—Paraffin section of myocardium from a mouse intoxicated with bromobenzene (15 mmol/kg body wt, by mouth) and sacrificed 20 hours later. Note the area of recent infarction. The necrotic portion of the myocardium appears paler than the surrounding muscle. (H&E, ×16)

extent than in the liver. Thus, an important feature of the present work is the realization that bromobenzene intoxication can lead to the development of lipid peroxidation probably in most tissues of the living body. To our knowledge, in fact, as far as the extrahepatic tissues are concerned, direct evidence for the occurrence of lipid peroxidation *in vivo* has been provided in only a few cases: these include peroxidation of lung lipids after exposure to nitrogen dioxide[24] and ozone[25-27] or after intoxication with the herbicide paraquat[28]; lipid peroxidation in experimentally induced brain edema[29]; and lipid peroxidation after reoxygenation of anoxic heart.[30,31] On the other hand, no lipid peroxidation could be demonstrated in lung,[32] kidney,[33,34] and intestinal mucosa[33] of rats poisoned with the prototype prooxidant carbon tetrachloride.

The GSH depletion induced by bromobenzene in the extrahepatic tissues can be explained by at least two mechanisms. One is that bromobenzene is metabolized in these tissues, as it is known to occur in the liver; and the reaction of the metabolites with GSH induces GSH depletion with its consequent effects. This mechanism may occur in lung (bronchiolar epithelium) and kidney (renal cortex). In fact, mixed function monooxygenase activity and cytochrome P-450 have been

Table 5—Glutathione (GSH) Depletion and Lipid Peroxidation (Malonic Dialdehyde Content, MDA) in Forebrain and Hindbrain After Bromobenzene Poisoning

	Time after intoxication	
	0 min	15–20 hours
GSH (μmol/g tissue)		
Forebrain	2.6 ± 0.1 (5)	1.6 ± 0.1* (15)
Hindbrain	2.0 ± 0.2 (5)	0.9 ± 0.04* (15)
MDA (nmol/g tissue)		
Forebrain	–	4.1 ± 1.3 (15)
Hindbrain	–	2.9 ± 1.1 (15)

Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Significantly different from the 0-minute value: $P < 0.002$.

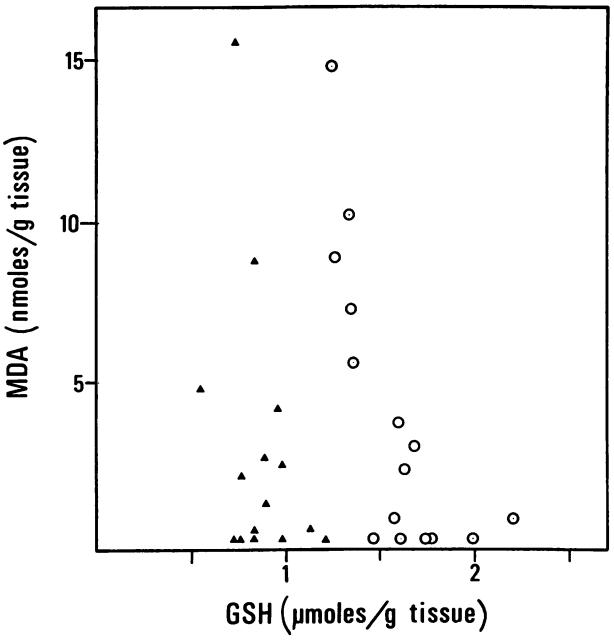

Figure 9—Plot of lipid peroxidation values (tissue contents of malonic dialdehyde) against the corresponding GSH contents in forebrain (O) and hindbrain (Δ) of mice intoxicated with bromobenzene (15 mmol/kg body wt, by mouth) and sacrificed 18–20 hours later.

demonstrated in the "Clara cells" of the bronchiolar epithelium[35-37] and in the renal cortex.[38,39] It must be noted, in addition, that within the first hours after bromobenzene administration the GSH depletion is very fast in lung and kidney and resembles that seen in liver; it is much slower in heart.

Another possible mechanism is postulated by some authors[40,41]: The metabolism of bromobenzene may occur mainly, if not exclusively, in the liver, and some metabolites, diffusing from the liver, reach extrahepatic organs. In these extrahepatic tissues some of these products would be able to react with GSH, causing its depletion and the consequent effects. Possibly the diffusing metabolites are transported in the form of precursors to extrahepatic tissues, where the precursors are converted to reactive metabolites. This second mechanism may apply to heart and other tissues, in which it is highly likely that the metabolism of xenobiotics does not occur. According to some reports,[15-18,40,41] at least part of bromobenzene-induced kidney and lung damage could also be explained in this way. In fact, phenobarbital pretreatment increased bromobenzene activation *in vitro* by liver microsomes but not by kidney[16] or lung[15] microsomes. However, phenobarbital pretreatment of mice given a toxic dose of bromobenzene strikingly increased the *in vivo* covalent binding of bromobenzene to kidney[16] and lung[15] proteins, in addition to liver proteins. This pretreatment also had similar effects on the *in vivo* covalent binding of bromobenzene to other extrahepatic tissues, such as heart and spleen,[15] in which microsomal mixed-function oxidase activities are virtually absent. These experiments indicated that a reactive bromobenzene metabolite formed in the liver is sufficiently stable to enter the circulation and travel to extrahepatic tissues, where it becomes covalently bound. This does not exclude the possibility that part of the covalently bound material derives from the local metabolism of bromobenzene in extrahepatic tissues, such as lung and kidney, which are provided with mixed-function oxidase activities.

In the experiments reported in Table 6, partially hepatectomized mice were given bromobenzene, and the GSH content of kidney and lung was assayed 3 hours thereafter. It was found that the GSH decrease was similar to that of the sham operation controls in lung and was lower in kidney. This result may be interpreted as indicating that in the partially hepatectomized animals there is a decreased supply of bromobenzene metabolites from liver to kidney, as well as an unaffected metabolism of bromobenzene in lung.

The appearance of lipid peroxidation in liver and extrahepatic tissues of bromobenzene-treated animals raises the question of how it is induced after bromo-

Table 6—Glutathione (GSH) Depletion in Lung and Kidney After Bromobenzene Poisoning of Partially Hepatectomized Mice

	GSH (nmol/mg protein)			
	Lung	% Decrease	Kidney	% Decrease
Sham operation				
Control	18.6 ± 2.3 (4)		23.6 ± 2.5 (4)	
Bromobenzene	14.5 ± 1.6 (7)	−25.1 ± 4.4* (7)	16.7 ± 1.7 (7)	−30.7 ± 2.8† (7)
Partial hepatectomy				
Control	19.2 ± 1.5 (4)		23.0 ± 2.8 (4)	
Bromobenzene	15.6 ± 1.1 (8)	−19.4 ± 2.6* (8)	19.1 ± 1.5 (8)	−17.4 ± 3.5† (8)

Partial hepatectomy was performed by exclusion of the left larger hepatic lobe. Bromobenzene was given by gastric intubation at the dose of 15 mmol/kg body wt immediately after partial hepatectomy. The animals were sacrificed 3 hours after poisoning. Results are given as means ± SEM. The number of animals is reported in parentheses.

* Difference not significant.

† $P < 0.05$.

benzene administration. An important feature of bromobenzene intoxication is the demonstration that, at least in some tissues, lipid peroxidation develops only when a threshold value of GSH is reached. This GSH threshold has been observed for liver,[14] kidney, skeletal muscle, and forebrain (present work). It is tempting to speculate that beyond a certain GSH concentration, the cell, whatever it may be, has no available defenses against a constitutive oxidative stress. Supporting evidence for such a view derives from the previous observation[14] that lipid peroxidation is induced in the liver by treating mice with diethylmaleate, which is mainly conjugated with hepatic GSH by glutathione-S-transferase activities without previous metabolism.[42] Recently, a protein heat-labile, GSH-dependent factor which is able to inhibit lipid peroxidation has been described in the cytosol of several cellular types of the rat.[43] This factor was shown to be distinct from both GSH peroxidase and GSH transferases. Thus, it is also conceivable that a fall in hepatic GSH content, such as that induced by bromobenzene in mice, would also impair this additional, as yet ill-defined, protective system.

An additional possibility is that lipid peroxidation is initiated as a consequence of the metabolism of bromobenzene in the tissues in which, as previously discussed, the metabolism of the toxin can occur. According to this hypothesis, which has been already discussed for the liver,[14] activated oxygen species produced during the metabolism of bromobenzene are the source of the oxidative stress responsible for the induction of lipid peroxidation. In isolated microsomes *in vitro*, production of active oxygen species has been shown to result from active turnover of the cytochrome P-450 system during mixed function oxidation.[44,45] It is conceivable that analogous phenomena can take place *in vivo* after oxygen-dependent metabolism of bromobenzene, thus leading to the appearance of lipid peroxidation in a liver cell whose antioxidant defenses are compromised by significant loss of GSH. On the other hand, the high levels of antioxidant defenses normally present in the liver cells imply that they may be exposed to significant oxidative stress even in the absence of drug-induced activation of the mixed-function oxydase system.

Previous studies[5-8,15,16,41] suggested that the cellular damage produced by bromobenzene in both liver and extrahepatic tissues is related to the covalent binding of reactive metabolites to cellular macromolecules. The present study, even if it cannot exclude this possibility, offers an alternative explanation for the bromobenzene-induced cellular damage. Bromobenzene induces lipid peroxidation in all the tissues examined, and this process seems to be related to the cellular injury. In fact, severe cellular damage occurs, at least in some tissues, only when the GSH depletion reaches a threshold limit and the same threshold value of GSH is in general evident for both the onset of lipid peroxidation and the development of cellular damage. GSH depletion and lipid peroxidation seem to be general phenomena occurring not only in the liver but in all the tissues as a consequence of bromobenzene poisoning. The possibility, therefore, exists that destructive lipid peroxidation is the cause of the structural alterations seen in liver and extrahepatic tissues after bromobenzene intoxication. The alternative possibility, that elevation of MDA level in tissues could result from peroxidative breakdown of injured cells, which are dead or dying as a consequence of a different mechanism, seems to us unlikely, at least in the model studied. In fact, as previously reported by us,[14] the treatment of mice after bromobenzene intoxication with the antioxidant Trolox C is able to prevent the onset of lipid peroxidation in liver, and concomitantly to suppress the elevation of SGPT levels. Moreover, a similar prevention of both lipid peroxidation in liver and elevation of SGPT has been observed in our laboratory (Casini et al, unpublished data) by treating the intoxicated animals with deferoxamine, an iron-chelating agent whose ability to prevent the initiation and/or propagation of the peroxidative process has been well documented.[46]

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